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Constitutive and inducible expression of interleukin-6 by Langerhans cells and lymph node dendritic cells

M. CUMBERBATCH, R. J. DEARMAN & I. KIMBER *Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK*

SUMMARY

During the induction phase of contact sensitization and other cutaneous immune responses a proportion of epidermal Langerhans cells (LC) is induced to leave the skin and migrate via afferent lymphatics to lymph nodes draining the site of exposure. The cells that accumulate in draining nodes have acquired the characteristics of immunostimulatory dendritic cells and effectively present antigen to responsive T lymphocytes. In the present study we have questioned whether LC in the epidermis and the lymph node dendritic cells into which they develop express interleukin-6 (IL-6), a cytokine that has been shown to serve as an important costimulator of T lymphocyte activation. *In situ* immunocytochemical analyses using a biotin–streptavidin staining technique revealed that dendritic cells resident in the epidermis of untreated mice constitutively express this cytokine. Keratinocytes expressed detectable IL-6 only following local exposure to the contact allergen oxazolone. Such treatment also appeared to enhance the expression by epidermal dendritic cells of this cytokine. Analyses of unfractionated and LC-enriched and -depleted populations of epidermal cells revealed a close correlation between major histocompatibility complex (MHC) class II (Ia) antigen expression and staining for IL-6, implicating LC as the sole or major source of this cytokine in unstimulated epidermis. Finally, compared with tissue isolated from mice treated with vehicle alone, draining lymph nodes prepared from animals 18 hr following sensitization with oxazolone displayed a substantial increase in both the frequency of dendritic cells and the number of IL-6$^+$ cells within the paracortex. These data demonstrate that resident epidermal LC and the dendritic cells into which they develop are important sources of IL-6. Their constitutive and inducible expression of this cytokine will facilitate the induction of cutaneous immune responses.

INTRODUCTION

Of central importance during the induction of cutaneous immune responses are the migration of antigen-bearing epidermal Langerhans cells (LC) from the skin and their accumulation, in an immunologically active state, in lymph nodes draining the site of exposure.[1–3] An early event during contact sensitization is the induction and upregulation of cytokine expression by epidermal cells.[4,5] Collectively these cytokines orchestrate immune and inflammatory responses in the skin and regulate the function of LC. There is evidence that tumour necrosis factor α (TNF-α), a keratinocyte-derived cytokine, provides a signal for LC migration from the epidermis,[6–8] and that another keratinocyte product, granulocyte–macrophage colony-stimulating factor (GM-CSF), acting in concert with other cytokines, effects the functional maturation of LC into potent immunostimulatory cells.[9,10] Other epidermal cytokines participate in the stimulation of cutaneous immune function and it has been demonstrated, for instance, that interleukin 1β (IL-1β), a product exclusively of LC in murine epidermis,[4] is necessary for contact sensitization.[11] Of relevance also is interleukin-6 (IL-6), a cytokine that displays multiple biological activities including, in association usually with IL-1β, costimulation of T lymphocyte activation.[12–15] Consistent with an important role for IL-6 in the stimulation of T lymphocyte responses during the induction phase of contact sensitization is the observation that the production by draining lymph node cell populations of this cytokine correlates closely with their proliferative activity.[16] It is apparent, however, that the primary source of IL-6 in allergen-activated draining lymph nodes is not T lymphocytes but dendritic cells (DC).[16,17] The question addressed in this paper is whether epidermal Langerhans cells themselves, as the precursors of many DC

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Abbreviations: AOO, 4:1 acetone:olive oil; DAB, diaminobenzidine; DC, dendritic cells; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1β, interleukin 1β; IL-6, interleukin 6; LC, Langerhans cells; LNC, lymph node cells; PBS, phosphate buffered saline; TNF-α, tumour necrosis factor α.

Correspondence: M. Cumberbatch, Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK.

found within activated skin-draining lymph nodes, are a source of constitutive or inducible IL-6 production. The expression of this cytokine by LC and lymph node DC *in situ* and by isolated cells has been examined immunocytochemically.

MATERIALS AND METHODS

Animals

Young adult (6- to 8-week old) BALB/c strain mice were obtained from Harlan Olac Ltd (Bicester, UK) and used throughout these studies.

Chemicals and exposure

The contact sensitizing chemical oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one; Sigma Chemical Co., St. Louis, MO) was used as a commercial preparation and dissolved in 4 : 1 acetone : olive oil (AOO). Groups of mice ($n = 3$ or $n = 10$) received 25 μl of the appropriate concentration of oxazolone on the dorsum of both ears. Control animals were treated with an identical volume of AOO alone.

Preparation of tissue sections

Skin sections were prepared from the ears of naive mice or from mice at various periods (2, 4, 6, 8, 12 or 24 hr) following topical exposure to 1% oxazolone or AOO ($n = 5$). Sections of draining (auricular) lymph nodes were prepared from mice exposed 18 hr earlier to 1% oxazolone or AOO. Fresh tissue samples were snap-frozen in isopentane (BDH Ltd, Poole, UK) cooled in liquid nitrogen, embedded in OCT compound (Tissue Tek, Raymond A Lamb Ltd, London, UK) and 6 μm frozen sections cut using a cryostat (Bright Instrument Company Ltd., Huntingdon, UK). Sections were transferred to microscope slides coated with poly-L-lysine (Sigma) and either examined immediately, or stored at $-70°$ prior to analysis.

Isolation of epidermal cells and enrichment for LC

Ears from naive mice were split with the aid of forceps into dorsal and ventral halves. Dorsal halves were incubated for 20 min at 37° in Hanks' Balanced Salt Solution containing 0·5% trypsin (Gibco Ltd, Paisley, UK). Epidermal sheets were removed and washed thoroughly in RPMI-1640 growth medium (Gibco) supplemented with 25 mM HEPES, 400 μg/ml streptomycin, 400 μg/ml ampicillin and containing 20% heat-inactivated fetal calf serum (FCS; Gibco). A single cell suspension of epidermal cells was prepared by gentle mechanical disaggregation through 200-mesh stainless steel gauze, washed twice in RPMI medium containing 20% FCS and finally resuspended in RPMI supplemented with 10% FCS (RPMI–FCS). Viable cell counts were performed by exclusion of 0.5% trypan blue and the cell concentration adjusted to 4×10^6 cells/ml. Populations enriched for LC were prepared according to the method of Streilein and Grammer.[18] Briefly, 5 ml of Lymphoprep (Nygaard, Oslo, Norway) were layered gently under 5 ml of epidermal cells and centrifuged (500 *g*) for 20 min at room temperature. Cells accumulating at the interface were collected, washed once and resuspended in RPMI–FCS. The frequency of major histocompatibility complex (MHC) class II $(Ia)^+$ LC in epidermal cell suspensions was measured using indirect immunofluorescence analysis of cytocentrifuge preparations.

Isolation of lymph node cells and enrichment for DC

Draining lymph nodes were excised 18 hr following exposure. Lymph nodes were pooled for each experimental group and a single cell suspension of lymph node cells (LNC) prepared by mechanical disaggregation through 200-mesh stainless steel gauze. LNC were washed and resuspended in RPMI–FCS, viable cell counts performed by exclusion of 0·5% trypan blue and the cell concentration adjusted to 5×10^6 cells/ml. Populations enriched for DC were prepared as described previously by Macatonia *et al.*[19] Briefly, 2 ml of Metrizamide (Nygaard; 14·5% in RPMI–FCS) were layered gently under 8 ml of LNC and tubes centrifuged (600 *g*) for 15 min at room temperature. Cells accumulating at the interface were collected, washed once and resuspended in RPMI–FCS. The frequency of DC in such low buoyant density fractions was measured routinely by direct morphological examination using interference contrast microscopy. Results are expressed as DC/node.

Cytocentrifuge preparations

Cell suspensions were adjusted to $1{\cdot}4 \times 10^5$ cells/ml in RPMI–FCS. Aliquots of 150 μl were loaded into cuvettes and centrifuged (800 *g*) for 6 min in a Cytospin cytocentrifuge (Shandon Ltd, Runcorn, UK).

Immunocytochemical analyses

All samples (both cytocentrifuge preparations and tissue sections) were fixed in acetone for 5 min at 4°. Prior to immunostaining, suspensions of epidermal cells, enriched LC suspensions and skin sections were treated for 5 min at room temperature with 3% hydrogen peroxide (30% solution; BDH Ltd) diluted in phosphate buffered saline (PBS; pH 7·2) to quench endogenous peroxidase activity. Material was then incubated for a further 20 min at room temperature sequentially with each of 0·01% avidin in PBS and 0·001% biotin in PBS to block endogenous avidin-binding activity. All washes were performed using PBS. To detect IL-6, samples were incubated overnight at 4° with rat anti-mouse IL-6 (IgG1 isotype; Genzyme Diagnostics, West Malling, Kent, UK) diluted to 10 μg/ml in PBS. LC and DC were identified following incubation for 30 min at room temperature with rat anti-mouse I-A antibody (IgG2b isotype; Serotec, Kidlington, UK) diluted to 2 μg/ml in PBS. Following incubation with primary antibodies, samples were washed for a total of 30 min in three changes of PBS and incubated at room temperature for 30 min with a biotin-conjugated $F(ab')_2$ fragment of rabbit anti-rat IgG (Serotec) diluted to 5 μg/ml with 10% normal mouse serum in PBS. Samples were then washed in three changes of PBS followed by a further incubation for 45–60 min with ExtrAvidin-peroxidase (Sigma) diluted 1 : 50 with PBS. Specific binding was detected with either diaminobenzidine (DAB; Sigma), producing a brown reaction product or with DAB containing 3% nickel chloride, producing a blue/black reaction product. Slides were washed in three changes of PBS, mounted in glycerol/saline (9 : 1) and sealed with nail varnish. Concurrent controls included the use of relevant isotype-matched antibody in place of the primary antibody (purified rat IgG1 and IgG2b rat anti-human HLA class I; Serotec). No staining was observed with any of the control samples.

RESULTS

Immunocytochemical analyses of skin sections, using a biotin–streptavidin immunoperoxidase staining technique, revealed that within the epidermis of naive mice a small proportion of cells displaying dendritic morphology expresses IL-6 (Fig. 1a). In naive mice no IL-6 was detected in adjacent keratinocytes, although a number of cells located in the dermis were clearly positive for this cytokine (Fig. 1a). Within 12 hr of exposure to a skin sensitizing concentration (1%) of oxazolone IL-6$^+$ epidermal dendritic cells exhibited signs of activation, manifest typically as a more dendritic morphology (Fig. 1b). Treatment was associated also with an apparent increase in the intensity of staining for IL-6 in dendritic cells which was maximal at 12 hr and had returned to control levels by 24 hr (Table 1). Treatment of mice with vehicle alone failed to influence the appearance, or IL-6 staining intensity, of dendritic cells within the epidermis at any time point examined (data not presented). Exposure of mice to oxazolone induced also the expression by keratinocytes of IL-6, as illustrated in Fig. 1b where skin was examined 12 hr following treatment. Keratinocyte expression of IL-6 was first detectable 6 hr following exposure to oxazolone, had reached maximal levels at 8 hr and had subsided by 24 hr. A subjective comparison of constitutive and inducible expression of IL-6 by epidermal dendritic cells and keratinocytes is summarized in Table 1. Following exposure to oxazolone cells throughout the dermis were found also to be contributing to the overall picture of increased IL-6 activity within the skin (data not presented). Analysis of skin sections prepared from mice treated with vehicle alone revealed that without exception there was no change in IL-6 expression in any skin compartment.

Additional experiments were conducted to characterize further the IL-6$^+$ epidermal dendritic cells. In addition to Ia$^+$ LC there exists within murine epidermis a second population of dendritic cells that lacks Ia, but which expresses instead the Thy-1 glycoprotein.[20,21] We have demonstrated previously that in BALB/c strain mice the frequency of dendritic epidermal T cells (Thy-1$^+$ DC) varies between 50 and 150 cells/mm^2, compared with between approximately 800 and 1100 cells/mm^2 for Ia$^+$ LC.[7] Serial cytospin preparations of epidermal cells isolated from naive mice were analysed for possession of Ia and for expression of IL-6. Equivalent frequencies (of between 2 and 4% in several independent experiments) of Ia$^+$ and IL-6$^+$ epidermal cells were identified. It was apparent, however, that the intensity of staining for IL-6 varied greatly between IL-6$^+$ cells. The expression of IL-6 by dendritic cells within an unfractionated epidermal cell population is shown in Fig. 1c. Similar analyses were conducted using preparations of epidermal cells that had been enriched for, or depleted of, LC by density gradient centrifugation. Although it was clear that LC-enriched populations of epidermal cells (comprising between 25 and 50% Ia$^+$ LC) contained a greatly increased frequency (25–50%) of IL-6$^+$ cells compared with unfractionated preparations, much weaker staining for the cytokine was observed. This reduced immunostaining for IL-6 almost certainly was the result of the extended enrichment procedure. Epidermal cell populations depleted of LC and containing < 1% Ia$^+$ cells had very few (< 1%) IL-6 staining cells (data not presented).

Topical exposure of mice to contact allergens causes the migration of LC from the epidermis and the accumulation of DC within draining lymph nodes.[1,2] We therefore examined whether skin sensitization with oxazolone resulted in the appearance of IL-6$^+$ DC in draining lymph nodes in the hours following exposure. Auricular lymph nodes isolated from naive BALB/c mice or BALB/c mice exposed to vehicle alone characteristically contain between 1×10^3 and 4×10^3 dendritic cells.[6] Treatment of the ears of mice with oxazolone causes a substantial increase in the frequency of lymph node DC that is most pronounced between 18 and 24 hr following exposure. Representative data recorded in six independent experiments conducted during the current series of investigations are shown in Table 2. Although in the experiments performed 18 hr following exposure to oxazolone or vehicle there was some variation in DC numbers, it is apparent from the data obtained that sensitization resulted in 4–5-fold increase in DC numbers within draining nodes by 18 hr and an approximately 7-fold increase by 24 hr.

Low buoyant density, DC-enriched fractions of draining LNC were examined for expression of IL-6 18 hr following exposure to oxazolone or vehicle alone. Consistent with previous investigations,[22] it was found that such populations comprised approximately 80% Ia$^+$ DC. As illustrated in Fig. 1d, the majority of lymph node DC were found to express IL-6 irrespective of whether mice had been treated with the

Table 1. Constitutive and inducible expression of IL-6 by epidermal dendritic cells and keratinocytes following exposure to oxazolone

	Staining intensity Time following exposure to oxazolone (hr)						
	0	2	4	6	8	12	24
Epidermal DC	++	++	++	++	+++	++++	++
Keratinocytes	−	−	−	+/−	+++	+	−

Summary of data based upon visual assessment of staining intensity for IL-6 in three independent kinetic experiments.

Table 2. Frequency of DC in draining lymph nodes following exposure of mice to oxazolone or AOO

Time following exposure (hr)	Experiment	DC/node		Fold increase
		AOO	OX	
18	1	4350	20475	4·7
	2	3829	16425	4·3
	3	1586	7031	4·4
24	4	2987	21201	7·1
	5	2920	21874	7·5
	6	3518	23461	6·6

Groups of mice (n = 10) received 25 μl of 1% oxazolone (OX) in AOO, or an equal volume of AOO alone, on the dorsum of both ears. Mice were sacrificed 18 or 24 hr following exposure and draining auricular lymph nodes excised. Single cell suspensions of LC were prepared and DC-enriched fractions isolated by density gradient centrifugation on Metrizamide. The frequency of DC was measured using interference contrast microscopy.

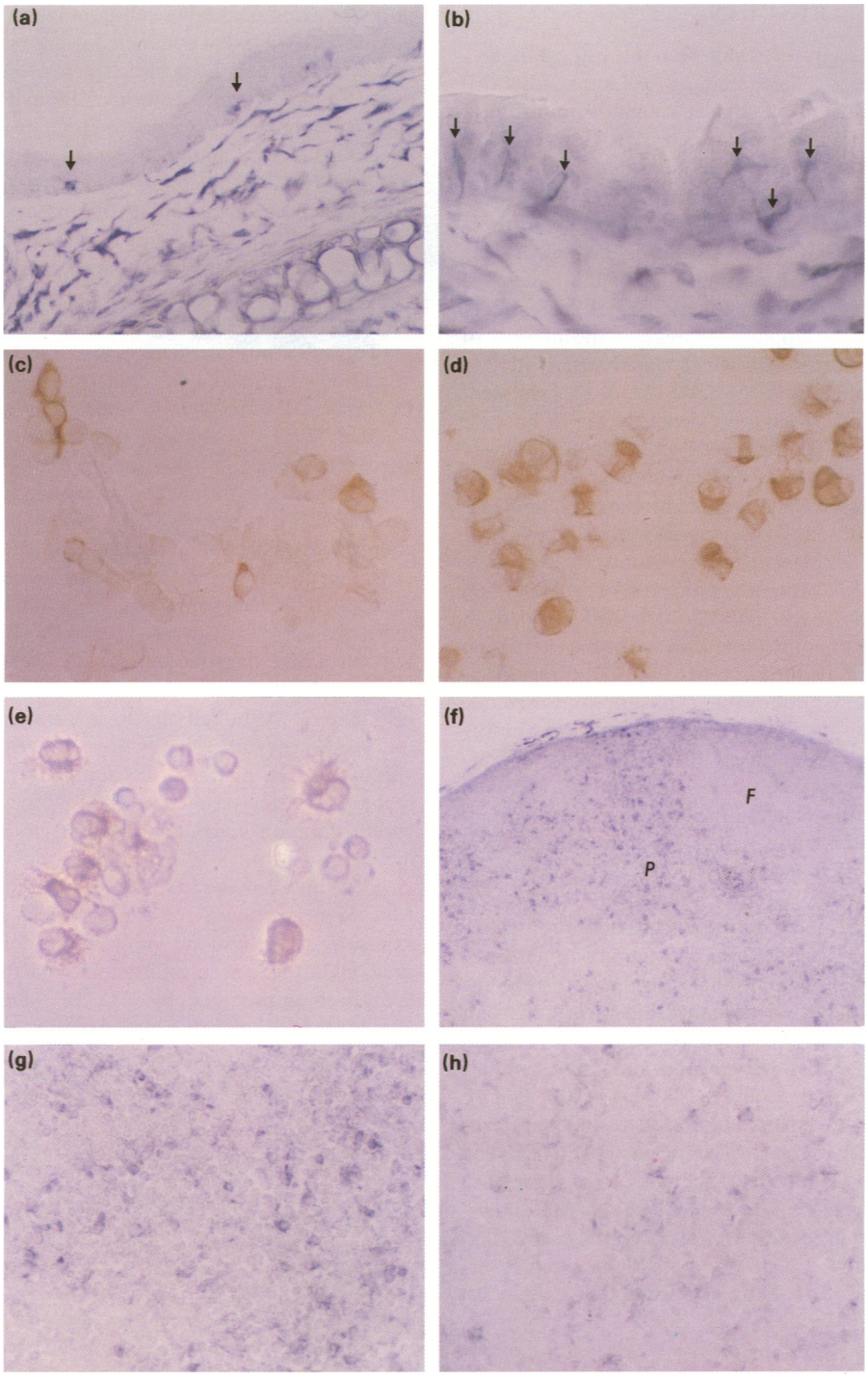

Figure 1. Immunodetection of IL-6$^+$ cells. (a) Section of naive skin revealing constitutive expression of IL-6 by epidermal DC (arrowed) (×250). (b) Section of skin prepared 12 hr following exposure to 1% oxazolone showing enhanced expression of IL-6 by epidermal DC (arrowed) and induced expression by keratinocytes (×400). (c) IL-6 expression by cells with dendritic morphology in epidermal cell suspensions prepared from naive mice (×400). (d) IL-6$^+$ DC within the low buoyant density fraction of LNC prepared from the draining nodes of mice exposed 18 hr previously to 1% oxazolone (×400). (e) Phase contrast micrograph of IL-6$^+$ DC within the low buoyant density fraction of draining LNC prepared from mice exposed 18 hr previously to 1% oxazolone (×400). (f) Localization of IL-6$^+$ cells within the paracortex (P), but not follicular areas (F) of draining lymph node sections prepared 18 hr following exposure of mice to 1% oxazolone (×100). (g) IL-6$^+$ cells within the paracortical region of draining lymph node sections prepared 18 hr following exposure of mice to 1% oxazolone (×250). (h) IL-6$^+$ cells within the paracortical region of draining lymph node sections prepared 18 hr following exposure of mice to AOO (×250).

contact allergen or vehicle. The dendritic morphology of cells present within the low buoyant density fraction of LNC was more apparent when analyses were performed using phase contrast microscopy (Fig. 1e). Thus, although the majority of DC found within draining lymph nodes prepared from both oxazolone- and AOO-treated mice expresses IL-6, the number of cytokine producing cells per node is greater in animals exposed to allergen (cf. Table 2). This is reflected by the results of parallel experiments in which IL-6$^+$ cells were identified in frozen sections of draining lymph nodes. It was found that IL-6$^+$ cells within the draining lymph nodes of both oxazolone and vehicle treated mice were located almost exclusively in the paracortex (Fig. 1f). At higher magnification it was clear that the frequency of IL-6$^+$ cells in the paracortical regions of draining lymph nodes was higher in oxazolone treated mice (Fig. 1g) than in mice treated with AOO alone (Fig. 1h).

DISCUSSION

The results described here reveal that murine epidermal LC constitutively express IL-6 protein and that such expression is augmented following topical application of a skin sensitizing chemical. It is apparent, however, that in normal skin, cells that reside in the dermis are also IL-6$^+$. Although a detailed evaluation of cells expressing IL-6 in the dermis was not undertaken, on the basis of previous investigations it is likely that endothelial cells, fibroblasts, macrophages and possibly dermal dendritic cells may be contributors to the overall pattern of IL-6 production in untreated skin.[23] These same cells, together with the induced expression by keratinocytes and apparently increased production by resident epidermal LC, may also provide for the enhanced local production in the skin of IL-6 following skin sensitization that is indicated by the data shown here.

While on the basis of *in situ* immunocytochemistry and the analysis of whole and fractionated populations of epidermal cells it is clear that LC produce IL-6 constitutively, it is not possible, despite the strong association between the expression of this cytokine and Ia, to exclude the possibility that some Thy-1$^+$ DC may also produce IL-6.[20,21] However, the available evidence indicates that the contribution of Thy-1$^+$ DC to epidermal IL-6 expression will be very limited. Matsue *et al.*[24] found that freshly isolated epidermal Thy-1$^+$ DC did not express IL-6. In the same studies it was demonstrated that stimulation of Thy-1$^+$ DC with concanavalin A and long-term culture resulted in a phenotype of expanded cytokine expression, including the appearance of mRNA for IL-6. The conclusion is that Thy-1$^+$ DC do not contribute significantly to either native or inducible levels of IL-6 production in the epidermis, although under conditions where such populations are activated and/or expanded then they may assume a greater significance.

This is the first demonstration that normal LC resident in the epidermis are able to produce IL-6. The only similar investigation was performed by Schreiber *et al.*[25] who observed that murine LC that had been cultured for 72 hours possessed mRNA for, and secreted, IL-6. We propose that LC constitutively produce this cytokine and that following local exposure to antigen and/or receipt of the stimulus to migrate such expression is increased. During the induction phase of contact sensitization a proportion of the LC resident at the site of exposure is induced to migrate via the afferent lymphatics and to accumulate as immunostimulatory dendritic cells in draining lymph nodes. Not unexpectedly therefore, it was found in these investigations that DC isolated from skin draining lymph nodes express IL-6 protein and that contact sensitization is associated with an increased frequency of IL-6$^+$ cells within the paracortex. The migration of IL-6$^+$ LC from the epidermis to regional lymph nodes following skin sensitization is supported by recent investigations of the production by allergen activated LNC of this cytokine. It was reported that the major source of IL-6 in activated lymph nodes was not the T lymphocyte, but rather dendritic cells characterized on the basis of their possession of the epitope recognized by the antibody 33D1.[16,17] Although these latter studies were performed with draining lymph nodes isolated from mice 72 hr following sensitization, at the time of the peak T lymphocyte proliferative response and maximal IL-6 secretion, the observations made are consistent with the conclusions drawn here.

It is relevant to consider these data in the broad context of the contribution made by LC to the initiation of cutaneous immune responses. The primary physiological role of LC in the epidermis is the recognition, internalization and processing of exogenous antigen.[18] Following receipt of the signal to migrate, provided by TNF-α,[6–8] LC lose the capacity to process antigen and acquire instead the characteristics of immunostimulatory dendritic cells. This functional maturation is associated with the elevated expression of those membrane determinants that are required for effective presentation of antigen to T lymphocytes, intercellular adhesion molecule-1,[26] Ia[27] and B7 costimulatory molecules.[28,29] However, in addition to membrane receptor–ligand interactions, activation of T lymphocytes requires costimulatory molecules, of particular importance being IL-1β and IL-6 that may often act in concert, as for instance in the stimulation of interleukin 2 receptor expression.[23] It has been established already that in murine epidermis LC represent the sole source of IL-1β and that expression of this cytokine is enhanced rapidly following skin sensitization.[4,30,31] There is indirect evidence available that topical exposure of mice to contact allergens results in the induced expression by draining lymph node cells of IL-1 bioactivity and that the relevant species of this cytokine is IL-1β from dendritic cells.[32] Taken together these data suggest that the functional maturation of LC induced following skin sensitization is characterized also by the upregulated expression of IL-1β and IL-6 and that these cytokines together facilitate effective presentation of the inducing allergen to responsive T lymphocytes in the draining lymph nodes.

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